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Title: Genetic characterization of influenza A viruses circulating in pigs and isolated in north-east Spain during the period 2006-2007

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Abstract: Swine influenza virus is one of the most important pathogens involved in the swine respiratory disease complex. Recent serological surveys showed a high prevalence of swine influenza strains belonging to the H1N1, H1N2 and H3N2 subtypes circulating in pigs in Spain. However, little is known about their genome sequence. Five swine influenza strains were isolated from some unrelated outbreaks occurred during 2006-2007, and their complete genome sequences were determined. Phylogenetic analysis revealed that they belonged to the lineages "Avian-Like" H1N1, "Human-Like" H3N2, and "Human-Like" H1N2, showing tight relationships with early or contemporary strains described in Europe. Notably, one virus of the H1N2 subtype showed genetic and antigenic divergence with the European contemporary strains or vaccinal strains of the same subtype, suggesting that some local and divergent clusters of the virus may pass unnoticed in routinary subtyping. Finally, analysis on the entire pattern of genome segments suggested that a second reassortment event could have influenced the evolution of that divergent H1N2 strain.

Suggested Reviewers:

Paolo Pasquali, DVM, PhD
Editor-in-Chief
Research in Veterinary Science

Bellaterra, Barcelona (Spain)

29 November, 2013

Dear Dr. Paolo Pasquali,

We would like to thank the referee for the comments and suggestions which we found very helpful. We hope you further consider the revised manuscript Nº. RVSC-13-658 " GENETIC CHARACTERIZATION OF INFLUENZA A VIRUSES CIRCULATING IN PIGS AND ISOLATED IN NORTH-EAST SPAIN DURING THE PERIOD 2006-2007" for publication in Research in Veterinary Science as original research paper.

In the manuscript, all the modifications suggested by the reviewer have been written in red, excluding references but including minor changes that are not within the suggestions from the reviewer. Please find our point-by-point responses below. In order to provide clarity, we have included the reviewer's comments in italics and our comments/answers just below in boldface.

We really appreciate the editor as well as the reviewer's comments and we tried to address most if not all the comments. Thank you very much

for considering the submission of our manuscript to Research in Veterinary Science and we look forward to receiving your final decision.

Sincerely yours,

Dr. María Montoya

On behalf of all authors

Editor's comments.

1. Please supply Supplementary Figure 3 (line 128) and Supplementary Tables 1 (Line 124) and 2 (line 157). Please also provide a separate text file containing legends for the 3 supplementary figures. These files do not appear to have been uploaded in the previous submission.

There is no Figure 3, it was a typing mistake. We changed "Figure 3" for "Figure 2" at line 210 and we supplied it with tables 1 and 2. The legends were supplied as separated text file.

2. Please check that the formatting of references matches the journal requirements, e.g., first letter of each word of journal titles should be capitals.

The Reference section was re-formatted following the journal requirement. Lines 311-446.

3. Minor changes to text:

Line 38, showed a high prevalence

We changed line 38 following the editor´s suggestion.

Line 40-41, needs rephrasing, something like: ".however, little genome sequencing of these viruses has been performed."

We redraft line 40-42.

Line 48, Notably, one virus.

We changed line 45 following the editor´s suggestion.

Line 64, Orthomyxoviridae

We changed line 61 following the editor´s suggestion.

Line 68, introduced into the viral genome

Lines 63-65 were redrafted following the editor´s suggestion.

Line 69, error prone viral replication

Lines 63-65 were redraft following the editor´s suggestion.

Line 76-77, for the pig industry but also for public health reasons.

We changed lines 68-69 following the editor´s suggestion.

Line 107, showing clinical signs of influenza at the time

Line 99 was changed following the editor´s suggestion.

Line 139, GenBank

Line 131 was changed following the editor´s suggestion.

Line 146, 8 CDS from SwIVs

Line 138 was redrafted taking into account the editor´s suggestion.

Line 175, "Figure 1, 1a, and 2a in the supplementary file". Please clarify as these numbers do not match annotation of figures.

The matching of the annotation in the figures was clarified changing lines 167-168 for "Figure 1a, 2a and figures 1a-f in the supplementary file."

Line 188, highly related

Lines 181-182 was changed following the editor´s suggestion.

Line 189, highly related

Line 182 was changed following the editor´s suggestion.

Line 197, The surface

Line 191 was changed following the editor´s suggestion.

Line 198, divergent

Line 192 was changed following the editor´s suggestion.

Line 206-207, please redraft this sentence for clarity ("Besides..")

We redrafted lines 200-201 following the editor´s suggestion.

Line 208, he 1990s

Line 202 was changed following the editor´s suggestion.

Line 209-210, in terms of either identity or genetic distance

We changed lines 203-204 following the editor´s suggestion.

Line 213, The divergent

Line 207 was changed following the editor´s suggestion.

Line 217-218, please redraft this sentence for clarity ("The antigenic.")

Following the editor´s suggestion, this sentence was redrafted in lines 210-214 in the new manuscript.

Line 218, mutations along the entire length

Line 213 was changed following the editor´s suggestion.

Line 233, related to SwIV

Line 225 was changed following the editor´s suggestion.

Line 234, Or particular importance

Line 226 was changed following the editor´s suggestion.

Line 244-245, showed a similar phylogeographic pattern

We changed lines 237-238 following the editor´s suggestion.

Line 246, in the epidemiology

Line 239 was changed following the editor´s suggestion.

Line 249, .exception, as the "human-like".

Line 242 was changed following the editor´s suggestion.

Line 259, European countries and the lack of closely related sequences is due to the limited sampling.

We changed lines 251-252 following the editor´s suggestion.

Line 260-262, please redraft this sentence as I am not clear what you mean.

Following the editor´s suggestion, this sentence was redrafted in lines 252-255 in the new manuscript.

Line 264, differently from the corresponding

We changed lines 256-257 following the editor´s suggestion.

Line 270, have been acting to generate.

We changed lines 262-263 following the editor´s suggestion.

Line 272, first half of the 2000s [or " between 2000 and 2005"]

Line 264 was changed following the editor´s suggestion.

Line 282, clarify "spitted"

The sentence of lines 273-274 was re-drafted following the editor´s suggestion.

Line 289-290, could have changed its antigenic characteristics, thereby influencing.

Lines 277-281 were redrafted following the editor´s suggestion.

Line 291, a large variation

Line 279 was changed following the editor´s suggestion.

Line 294, If correct, this suggests that there .

Lines 281-282 were changed following the editor´s suggestion.

Line 297-298, serological testing might be useful to support the bioinformatics data.

We changed line 285 following the editor´s suggestion.

Reviewer #1: Title: Genetic characterization of influenza A viruses circulating in pigs and isolated in North-east Spain during the period 2006-2007.

Manuscript Overview

This study examines the genetic characteristics of H1N1, H1N2 and H3N2 influenza subtypes found in Spanish pigs. Overall, this paper is well-written and provides a contribution to the study of influenza viruses in Spain. Some grammatical errors and misspellings should have been corrected for better cascade of ideas.
While the data is of general interest, this reviewer recommends few revisions and clarifications of the manuscript prior to publication.

1. The objective of this study was clearly made apparent in the Abstract. However, it could have been more emphasized if the statements are less wordy.

The abstract section was redrafted taking into account the reviewer´s suggestion. Lines 37-50.

2. The Introduction can be made more concise i.e.page 4, from line 68 to 73 can be shortened by merely mentioning the types of genetic change in influenza virus genome that give rise to variant strains instead of defining each of them.

This paragraph in the introduction was redrafted taking into account the reviewer´s suggestion. Lines 63-65.

3. The Results section was very descriptive. However, more appropriate words need to be used in some areas for clarity; most specifically in the Molecular characterization section (page 10, line 213-221): "may have been changing" to "may have changed.","differently related" to "unidentical with." and "On the contrary" to "In contrast" etc.

The above sentences were modified following the reviewer´s suggestion. The introduction of these changes in the text suggested by the editor in this part was helpful in this results section. These sentences were modified in the new manuscript at lines 207-208, 211 and 214.

4. The Discussion part (page 11-12) could have been more interesting if the hypotheses were straightforward. In addition, the authors could have

refrained from repeating the items that were already mentioned in Methods/Results.

The section was redrafted following the reviewer´s suggestion. Lines 219-285.

5. Line 230: Instead of mentioning the specific isolate name, it would be better to come up with a more general term for the viruses represented by SF11131 to give a broader view of the genetic drift that occurred between SwIV strains in Europe.

The sentence was redrafted trying to follow the suggestions of the reviewer. Lines 223-224.

6. Line 238: Please clarify the statement, ".could suggest a direction of spreading." Did you mean, ".data give us clues on where the virus originated and the direction to which it spread"?

The suggestion of the reviewer was used to better clarify the sentence at lines 230-231.

7. Line 286: ".handicaps" can be changed to "...challenges".

We changed the line 274 following reviewer´s suggestion.

8. Line 297-298: Whose or which bioinformatics data?

We clarified the sentence at line 285.

9. The Conclusion section contains vague statements, i.e. line 302-304. Please clarify what you meant by "the opposite situation in which some viruses persist."

Conclusions section, at lines 288-295, was redraft with more concise statements.

10. In Figure Legends and Table Notes: Please change "bolded character" to boldface."

The cited changes were introduced as suggested by the reviewer. Lines 454, 464 in the manuscript and in the Table 4 notes.

Genetic characterization of influenza A viruses circulating in pigs and isolated in north-east Spain during the period 2006-2007

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Abbreviations:

HA: Hemagglutinin

NA: Neuraminidase

PB1: Polymerase basic protein 1

PB2: Polymerase Basic protein 2

PA: Polymerase acidic protein

NP: Nucleocapsid protein

M: Matrix protein

NS: Nonstructural protein

Abstract

Swine influenza virus is one of the most important pathogens involved in the swine respiratory disease complex. Recent serological surveys showed a high prevalence of swine influenza strains belonging to the H_1N_1, H_1N_2 and H_3N_2 subtypes circulating in pigs in Spain. However, little is known about their genome sequence. Five swine influenza strains were isolated from some unrelated outbreaks occurred during 2006-2007, and their complete genome sequences were determined. Phylogenetic analysis revealed that they belonged to the lineages "Avian-Like" H_1N_1, "Human-Like" H_3N_2, and "Human-Like" H_1N_2, showing tight relationships with early or contemporary strains described in Europe. Notably, one virus of the H_1N_2 subtype showed genetic and antigenic divergence with the European contemporary strains or vaccinal strains of the same subtype, suggesting that some local and divergent clusters of the virus may pass unnoticed in routinary subtyping. Finally, analysis on the entire pattern of genome segments suggested that a second reassortment event could have influenced the evolution of that divergent H_1N_2 strain.

Keywords: Spain, Swine influenza A virus, Sequencing

Introduction

Swine influenza virus (SwIV) is one of the viral pathogens involved in respiratory disorders in pigs, and it is also considered a contributor to the so-called porcine respiratory disease complex (Hansen et al., 2010). Furthermore, respiratory lesions are prevalent in pigs at slaughterhouse (Fraile et al., 2010). Continuous surveillance of SwIV infection is of great relevance for the pig industry, both to monitor the evolution of a rapidly changing virus, and to improve the measures adopted to control the disease during pig rearing. Swine influenza virus is a member of the *Orthomyxoviridae* family classified as type A (IAV). The viral genome is composed of eight single-stranded, negative-sense RNA segments. Evolution of RNA virus is described by the quasispecies theory, in which mutation (drift) and reassortment events (shift) have relevant roles (Domingo et al., 2012).

The pig has been considered as a "mixing vessel" for IAV, because it supports replication of avian and human influenza viruses (Altschul et al., 1990), thus the surveillance of SwIVs is of great relevance not only for the pig industry but also for public health reasons. During 2001-2004, our group and others reported the H_1N_1, H_3N_2 and H_1N_2 SwIVs as endemic in the pig population in Spain, with seroprevalences slightly lower than those described in other pig rearing areas in Europe (Maldonado et al., 2006; Van Reeth et al., 2008). The three subtypes were reported to co-circulate in pigs in Spain, with sows often being seropositive to multiple subtypes at a time, probably as a result of multiple contacts during life (Kyriakis et al., 2013; Maldonado et al., 2006). SwIVs described in the 2000s in Spain belonged to the "Avian-like" H_1N_1 (or better European Avian-Like), the "Human-Like" H_3N_2, and the "Human-Like" H_1N_2 lineages. Although described at the subtype level using partial sequencing and antigenic procedures, a low number of complete genetic sequences of SwIVs in Spain were

available for genetic comparison after that period. The three lineages were showed to be maintained in Spain from up to 2007 (Kyriakis et al., 2011; Kyriakis et al., 2013; Maldonado et al., 2006), although a new genetic lineage having an "Avian-Like" hemagglutinin gene related to the H_1N_1 strains circulating in Spain was circulating in the same period (Maldonado J., 2010; Valls, 2011). During 2009 the emergence of the pandemic IAVH_1N_1 (pdm09) changed dramatically the epidemiology of IAV globally. The new virus was able to infect and transmit among pigs (Brookes et al., 2009; Busquets et al., 2010), and quickly spread across pig populations in several European countries (Grøntvedt, 2011; Hjulsager, 2011). After the initial outbreaks, new reassortants of the pdm09 strain circulating in pigs were described in several countries worldwide (Moreno et al., 2011), making the epidemiology of IAV more complex.

The complete genetic characteristics of some SwIVs isolated during the pre-pandemic period 2006- 2007 were determinate in this work, fulfilling the lack of information describing their history and evolution. These data extend and complement the serological study already available in literature on that period.

Materials and methods

Virus isolation and subtype determination

Lungs were collected from freshly dead or euthanized pigs housed in 5 unrelated pig herds showing clinical signs of influenza at the time of sampling (Table 1). Virus isolation was attempted by inoculating the lung homogenates into the chorioallantoic sac of 9- to 11-day-old embryonated SPF chicken eggs. After 3-5 days of incubation at 37ºC, the allantoic fluids were tested for the presence of influenza virus by RT-PCR (Fouchier et al, 2000), and hemagglutination. Isolates were then tested by multiplex RT-

PCR (Chiapponi, 2003) and standard cross-hemagglutination inhibition to determine the virus subtype and lineage. Virus isolates were then propagated a maximum of 2 times on the Madin-Darby canine kidney (MDCK) cell line and stored at -80ºC until further testing.

Genome sequencing

Total RNA was extracted from the infected MDCK supernatants using the QIAamp® Viral RNA Mini kit (Qiagen), according to the manufacturer's instructions. The full length coding sequences (CDS) of the 8 segments of the SwIV isolates was obtained using an RT-PCR method, which combines several approaches listed below. Primers from the World Health Organization (2009) and from Chiapponi *et al*. (2003) were used. Additional primers were designed to obtain sequences of regions not amplified by the above cited sources (Table 1 in supplementary file). They were designed so they could be used under the same amplification conditions described in the WHO protocol. The AccessQuick™ master mix RT-PCR System (Promega) was used according to the conditions suggested by the WHO (detailed in Table 1 of Supplementary file). Amplification products were separated by standard agarose gel electrophoresis, and then purified using the NucleoSpin® Extract II (Macherey-Nagel) purification kit as per manufacturer's instructions. Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing kit v3.1 (Applied Byosystems), and resolved by using the ABI 3730 DNA automatic sequencer (Applied Biosystems). ChromasPro software was used to assemble and edit the overlapping fragments sequences (Technelysium Pty Ltd.).

Sequence analysis

BLAST sequence analysis (Altschul et al., 1990) was used for assessing the greatest similarity for each gene. Publicly available nucleotide sequences of IAVs were collected from the Influenza Virus Resource database (Bao et al., 2008) and GenBank. Sequences were aligned using the ClustalW (Thompson et al., 1994) tool implemented in the MEGA5 software (Tamura et al., 2011). A first phylogenetic tree for each CDS was constructed using all available sequences isolated in swine in Europe, with complete CDS. Then, for clarity, a second tree was constructed using only selected SwIV sequences representative of each cluster in the tree, along with all the existing sequences from Spanish isolates (available in the databases up to 14/03/2013). Phylogenetic trees of the eight CDS from SwIVs were constructed using MEGA5 software. The evolutionary history of SwIVs was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) with the Jukes-Cantor model (Jukes et al., 1969), and boostrapped 1000 times (Joseph, 1985). The reliability of the phylogenetic history was checked by comparing the phylograms generated with the above described method, and the phylograms generated with Maximum likelihood method based on the best DNA evolutionary model for each segment.

Screening for antigenic and glycosylation sites

The amino acid sequences of the hemagglutinin (HA) belonging to both Spanish field isolates, and strains in RESPIPORC FLU3/GRIPOVAC 3 (IDT Biologika GmbH, Merial S.A.S.) (Table 2 in supplementary file) were aligned and analysed for similarity (Figures 2 and 3 in supplementary file). The position of the antigenic sites Ca, Sa, Cb and Sb on the H1 type of the HA protein, were deduced from Brownlee 2001 (Brownlee and Fodor, 2001). The glycosylation motifs were identified by NetNGlyc 1.0 server (Gupta, 2004).

Results

The five isolated viruses belonged to the subtypes H_1N_1 (n=2), H_1N_2 (n=1) and H_3N_2 (n=2). The phylogenetic trees constructed from the full coding regions of the eight viral genomic segments, showed the same topology using either the Neighbor-Joining or the Maximum likelihood method (data not shown).

The internal genes (PB1, PB2, PA, NP, M and NS) CDSs of the isolated viruses belonged to the "Avian-Like" lineage and clustered together with SwIV strains isolated after 2004 (Table 2 and Supplementary files), whereas the surface glycoproteins CDSs (HA and NA) belonged to different lineages depending on the subtype.

H_1N_1 subtype

The phylogenetic analysis showed that all eight CDSs of the SwIV strain SF11131 belonged to the European "Avian-Like" lineage (Figures 1a, 2a and figures in the supplementary file 1 a-f) and clustered together with the contemporary strains isolated after 2000. This isolate also showed high identity (Table 2) and close clustering of the HA and NA CDSs, with the Spanish strain A/Swine/Spain/53207/2004 (H_1N_1). This latter, in turn, showed similar relationship with other European strains like A/Swine/Hungary/19774/2006 (H_1N_1), and A/swine/Germany/SIV04/2008 (H_1N_1) for HA CDS and A/swine/Italy/53949/2004 (H_1N_1) and again A/Swine/Hungary/19774/2006 (H_1N_1) for the NA CDS.

H_3N_2 subtypes

The HA and NA CDSs of the isolates SF32071 and 80598LP1 clustered with the European strains of the "Human-Like" H_3N_2 SwIV subtype. Both isolates exhibited a

close relationship with the Spanish strains A/Swine/Spain/54008/2004 (H_3N_2) and A/Swine/42386/2002 (H_3N_2), and clustered together with strains described in North-East Europe in 2006-2007.The HA CDS of isolate SF32071 was particularly highly related with A/swine/Spain/82108/2007 (H_3N_2), whereas the NA was highly related with the same strain but also with A/swine/Gent/96/2007 (H_3N_2) (Table 2, Figures 1b and 2b). Conversely, the HA CDS of isolate 80598LP1 exhibited a higher phylogenetic distance from SF32071 and the closest neighbour was the European strain A/swine/Gent/96/2007 (H_3N_2). The NA of 80598LP1 showed the same trend as the HA CDS but the closest neighbour was the Spanish strain A/Swine/Spain/54008/2004 (H_3N_2).

H_1N_2 Subtypes

The surface glycoproteins CDSs of the isolates SF12091 and 80598LP4 belonged to the same lineage of SwIVs, the "Human-like" H_1N_2. However, they showed a divergent evolution between them, which was particularly evident when analyzing the NA gene (Fig. 2b). The HA CDS of the isolate SF12091 was related to the Spanish strain A/Swine/Spain/40564/2002 (H_1N_2) and strains circulating in north-east Europe. Among them, the strain A/swine/Kitzen/IDT6142/2007 (H_1N_2) was the most closely related one (Table 2, Figure 1b). The NA CDS showed a clustering pattern similar to the HA CDS (Figure 2b).

The HA of isolate 80598LP4 was more phylogenetically distant from the other co-circulating H_1N_2 viruses, including the co-isolated SF12091. Besides, the greatest identity found by BLAST was 94%, the lowest compared to the other strains (Table 2, Figure 2b). On the contrary, the NA CDS clustered with strains circulating in the 1990s

in England and France. However, none of them showed a close relationship in terms of either identity or genetic distance (Table 2, Figure 1b and 2b).

Molecular characterization

The divergent evolution of the two H_1N_2 isolates SF12091 and 80598LP4 may have changed their antigenic characteristics; hence, amino acid sequences of their HA protein were compared between them and with the vaccine strain A/Swine/Bakum/1832/2000 (H_1N_2) which has a "Human-Like" HA (Supplementary file Figure 2). The antigenic sites of the two field isolates were unidentical with the vaccine strain. While the isolate SF12091 showed few mutations falling into the antigenic sites, the isolate 80598LP4 showed more mutations along the entire length of the protein but also in the hypothetical antigenic site Ca (Table 3). In contrast, the predicted glycosylation pattern was completely conserved among the H_1N_2 strains screened (Table 4), suggesting that the masking of the antigenic sites was conserved.

Discussion

Field infections of pigs with different SwIV subtypes were detected in Spain in this study, during the period 2006–2007. Five strains were isolated from finishing pigs, and their complete genomes were sequenced and analysed. The isolates belonged to the subtypes "Avian-Like" H_1N_1, "Human-Like" H_3N_2 and "Human-Like" H_1N_2.

The studied isolates shared a common origin with Spanish strains described previously in each subtype. Notably the H_1N_1 isolate could be considered drifted from one of them. Additionally, the viruses isolated in this study were also related to SwIV strains circulating in Germany, Hungary and Italy during 2004-2008. Of particular importance were the tight genetic relationship of some strains co-circulating during 2006-2007 (like

228 the isolate SF12091 with the German strain) and the contemporary surge of herds
229 affected by this subtype in Belgium or Italy after the outbreaks in Spain (Kyriakis et al.,
230 2013). These data give clues on where the virus originates and the direction to which it
231 spreads. However, further sequencing data on those foreign outbreaks would support
232 this hypothesis.
233 Comparisons among European and Spanish strains of SwIVs showed that genetically
234 similar viruses were causing outbreaks contemporarily in different countries, suggesting
235 that they could have been spread across the country in a short period of time. The
236 transboundary movement of live animals may partly explain this finding, because other
237 pig pathogens that spread in Spain in the same period showed a similar phylogeographic
238 pattern (Allepuz et al., 2007; Reuter et al., 2010). Transnational trade of live animals is
239 a relevant factor in the epidemiology of pigs diseases (Allepuz et al., 2007; Allepuz et
240 al., 2009; Mortensen et al., 2002; Rose and Madec, 2002; Zepeda et al., 2001), and
241 probably it might also be relevant in the case of SwIV. However, the isolate 80598LP4
242 was an exception, as the "Human Like" H_1N_2 strains were introduced in Europe from
243 England, with a second wave of introductions in France during '90s (Marozin et al.,
244 2002). The NA phylogeny suggests that isolate 80598LP4 might have evolved from the
245 latter. On the other hand the phylogeny of the HA did not show such clear clustering.
246 Albeit the origin of those genes coding for surface glycoproteins was far from clear and
247 consistent between them, it is noteworthy that they were divergently evolving from the
248 co-circulating H_1N_2 strains, and none of them showed a close relationship. This might
249 suggest that these viruses may have been persistently perpetuated in Spain, evolving
250 divergently than other circulating strains. It cannot be excluded, however, that the
251 isolate 80598LP4 could have been an external introduction from other European
252 countries, and the lack of closely related sequences is due to the limited sampling. Thus,

253 the persistence or the periodical re-introduction of the H_1N_2 subtype at regional level
254 has been recently reported by a serological study in five European countries included
255 Spain (Kyriakis et al., 2013).
256 The "internal genes" of the isolate 80598LP4 could have been evolved differently from
257 the corresponding surface glycoproteins coding genes. They belonged to the "Avian-
258 Like" lineage, as commonly observed in the "Human-Like" H_1N_2 viruses circulating in
259 Europe. However, they were highly related to both co-circulating H_1N_1 and H_1N_2. This
260 particular co-evolution of the 80598LP4 internal genes with the rest of the analyzed
261 H_1N_1, H_1N_2 and H_3N_2 viruses, together with the controversial divergent evolution of the
262 genes coding for the surface glycoprotein, suggested that a reassorting event could have
263 been acting to generate a "second generation" of reassortants.
264 The detection of an H_3N_1 SwIV in Spain during the first half of the 2000s (Martinez,
265 2008) suggested that reassortment was not an uncommon event. A great variety of new
266 reassortants have been described in different European countries, suggesting that
267 reassortment in SwIV is also a common event in other European pig rearing areas in the
268 continent (Moreno et al., 2009; Moreno et al., 2012; Zell et al., 2008a; Zell et al.,
269 2008b). The most important finding supporting this hypothesis was that the
270 reassortment of H_1N_1 and H_1N_2 subtypes had the highest rate (every 2-3 years) (Lycett
271 et al., 2012); thus, the origin of the internal genes of the isolate 80598LP4 could have
272 followed that trend.
273 The vaccines against swine influenza commercialized in Europe contain inactivated
274 SwIVs of the subtypes H_1N_2, H_3N_2 and H_1N_1. Antigenic drift is a one of the challenges
275 for the control of the disease in pigs using this kind of vaccines (Heinen et al., 2001;
276 Kyriakis et al., 2010; Van Reeth et al., 2001; Van Reeth et al., 2003; Zell et al., 2008b).
277 Thus, due to the divergent genetic evolution of the HA of the H_1N_2 isolated viruses, the

antigenic variations were evaluated comparing the most updated commercial vaccine. The isolate 80598LP4 showed a large variation of the hemagglutinin protein falling into but also outside of the hypothetical antigenic site. However the glycosylation pattern was the same, suggesting that at least the exposed antigenic sites were conserved. If correct, this suggests that there was an ongoing divergent evolution that changed the supposed antigenic sites in comparison to some other co-circulating strains; however, those results could not specify the effect on the serological recognition. Thus, further serological testing might be useful to support our bioinformatics data.

Conclusions

The genetic characteristics of some SwIV circulating in Spain during the period 2006-2007 showed some clues about transboundary introduction or reintroduction of SwIV in Spanish pig herds, being a common event in that period. However, this study also suggests that SwIV outbreaks could be due to the persistency of some viruses in determinate Spanish regions. The factors leading this mixed situation (introduction-reintroduction and persistency of SwIV strains) should be deeply investigated as they may help understanding the epidemiology of SwIV after pandemic outbreaks. Additionally, it may help establishing control plans of the disease in the future.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

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304 Spanish Ministry of Science and Innovation.
305
306 Appendix A. Supplementary material
307 Supplementary data associated with this article can be found in the online version, at
308 doi:….
309
310 **References**

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Figure legends

Figure 1: Evolutionary relationships of HA CDS of taxa. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap values greater than 50% are shown (1000 replicates). Strains isolated in this study are in boldface and Spanish SwIV strains previously isolated are underlined. a) H1 HA phylogenetic tree. The analysis involved 38 nucleotide sequences of H_1N_1 and H_1N_2 IV subtypes. There were a total of 1676 positions in the final dataset. b) H3 HA phylogenetic tree. The analysis involved 25 nucleotide sequences of H_3N_2 IV subtype. There were a total of 1694 positions in the final dataset

Figure 2: Evolutionary relationships of taxa of NA CDS. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap values greater than 50% are shown (1000 replicates). Strains isolated in this study are in boldface and Spanish SwIV strains previously isolated are underlined. a) N1 NA phylogenetic tree. The analysis involved 27 nucleotide sequences of H_1N_1 IV subtypes. There were a total of 1403 positions in the final dataset. b) N2 NA phylogenetic tree. The analysis involved 34 nucleotide sequences of H_3N_2 and H_1N_2 IV subtypes. There were a total of 1398 positions in the final dataset.

Supplementary files figures legends:

Figure 1: Evolutionary relationships of internal genes of taxa. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap values greater than 50% are shown (1000 replicates): a) NP phylogenetic tree. The analysis involved 53 nucleotide sequences. There were a total of 1462 positions in the final dataset; b) M phylogenetic tree. The analysis involved 52 nucleotide sequences. There were a total of 965 positions in the final dataset; c) PB2 phylogenetic tree. The analysis involved 42 nucleotide sequences. There were a total of 2275 positions in the final dataset. d) PB1 phylogenetic tree. The analysis involved 47 nucleotide sequences. There were a total of 2268 positions in the final dataset; e) PA phylogenetic tree. The analysis involved 46 nucleotide sequences. There were a total of 2148 positions in the final dataset; f) NS phylogenetic tree. The analysis involved 49 nucleotide sequences. There were a total of 830 positions in the final dataset.

Figure 2: Alignment of the H1HA protein of the H_1N_2 isolates SF12091 and 80598lp4 and the H_1N_2 strain composing the vaccine Respiporc flu3. The signal peptide is located at the firsts 16 aminoacid. The post traductional cleavage of the HA0 protein at the site 344 gives rise to the two subunit composing the HA protein: the HA1 (from position 18 to 343) and the HA2 (from position 345 to 566). The globular head of HA protein is located from position 59 to 292 in the HA1 subunit, on the contrary the stalk is located at the flanking regions. The transmembrane region is located from position 529 to 554 at the HA2 subunit. The antigenic sites Cb, Sb, Ca and Sa were identified on the alignment and highlighted in yellow. Relevant signatures in Receptor Binding site were highlighted with asterisks. Glycosylation site were identified at positions 27, 28, 40, 172, 177, 286, 304, 498, 557 (empty squares).

Table 1

Table 1: Data regarding the Spanish SwIV strains analyzed in this study.

Virus	**A/Swine/Spain/SF11131/2007 (H1N1)**	Abbreviation: **SF11131**
Outbreak	Location: Alcarras (Lleida)	
	Date: December 2007	
	Animals age[a]: 12	
	Mortality rate[b]: 3.28%	
Sampling date:	n/a	
Accession numbers:	HA: HF674888, NA: HF674889, PB2: HF674895, PB1: HF674894, PA: HF674893, NP: HF674891, M: HF674890, NS: HF674892	
Virus	**A/Swine/Spain/SF32071/2007 (H3N2)**	Abbreviation: **SF32071**
Outbreak	Location: Termens (Lleida)	
	Date: July 2007	
	Animals age: 20	
	Mortality rate: 3.07%	
Sampling date:	September 2007	
Accession numbers:	HA: HE774666, NA: HE774670, PB2: HE774669, PB1: HE774671, PA: HE774668, NP: HE774667, M: HE774673, NS: HE774672	
Virus	**A/Swine/Spain/80598LP1/2007 (H3N2)**	Abbreviation: **80598LP1**
Outbreak	Location: Torres de Segre (Lleida)	
	Date: August 2006	
	Animals age: 20	
	Mortality rate: 9.19%	
Sampling date:	May 2007	
Accession numbers:	HA: HF674896, NA: HF674897, PB2: HF674903, PB1: HF674902, PA: HF674901, NP: HF674899, M: HF674898, NS: HF674900	
Virus	**A/Swine/Spain/ SF12091/2007 (H1N2)**	Abbreviation: **SF12091**
Outbreak	Location: Binefar (Huesca)	
	Date: August 2007	
	Animals age: 22-24	
	Mortality rate: 6.99%	
Sampling date:	n/a	
Accession numbers:	HA: HF674904, NA: HF674905, PB2: HF674911, PB1: HF674910, PA: HF674909, NP: HF674907, M: HF674906, NS: HF674908	
Virus	**A/Swine/Spain/80598LP4/2007 (H1N2)**	Abbreviation: **80598LP4**
Outbreak	Location: Almenar (Lleida)	
	Date: September 2006	
	Animals age: 10-12	
	Mortality rate: 6.98%	
Sampling date:	May 2007	
Accession numbers:	HA: HF674912, NA: HF674913, PB2: HF674919, PB1: HF674918, PA: HF674917, NP: HF674915, M: HF674914, NS: HF674916	

[a] weeks of life

[b] average mortality during the fattening period

Table 2

Table 2: The most homologous SwIV sequences for each segment analyzed in this study, according to BLASTn search (25/02/2013).

SF11131	Identity	Strain	Accession number
HA	98%	A/swine/Spain/53207/2004(H1N1)	CY010580.1
NA	99%	A/swine/Italy/65296/2004(H1N1)	EU045389.2
		A/swine/Italy/53949/2004(H1N1)	EU045388.2
		A/swine/Spain/53207/2004(H1N1)	CY010582.1
PB1[b]	98%	A/swine/Spain/53207/2004(H1N1)	CY010586.1
		A/swine/Italy/71251/2005(H1N2)	JX843273.1
PB2	99%	A/swine/Gent/132/2005(H1N1)	CY116431.1
		A/swine/Spain/53207/2004(H1N1)	CY010587.1
		A/swine/Italy/50568/2005(H1N2)	HQ845023.1
PA	99%	A/swine/Spain/53207/2004(H1N1)	CY010585.1
		A/swine/Italy/50568/2005(H1N2)	HQ709221.1
NP[b]	99%	A/swine/Gent/132/2005(H1N1)	CY116435.1
		A/swine/Spain/53207/2004(H1N1)	CY010583.1
	98%	A/swine/Hungary/13509/2007(H3N2)	FJ798771.1
		A/swine/Greven/IDT2889/2004(H1N1)	GQ161157.1
M[b]	99%	A/swine/Spain/53207/2004(H1N1)	CY010581.1
		A/swine/Ploufragan/0214/2006(H1N2)	CY116542.1
		A/swine/Gent/132/2005(H1N1)	CY116437.1
NS	98%	A/swine/Spain/53207/2004(H1N1)	CY010584.1
		A/swine/Italy/159870-2/2005(H1N1)[a]	CY116483.1
		A/swine/Hungary/13509/2007(H3N2)[a]	FJ798774.1
SF32071			
HA	99%	A/swine/Spain/82108/2007(H3N2)	CY116558.1
	98%	A/swine/Hungary/13509/2007(H3N2)	FJ798772.1
		A/swine/Damme/IDT5673/2006(H3N2)[a]	GQ161147.1
		A/swine/Spain/54008/2004(H3N2)	CY010564.1
NA	99%	A/swine/Spain/82108/2007(H3N2)	CY116560.1
		A/swine/Gent/96/2007(H3N2)	CY116452.1
		A/swine/Hungary/13509/2007(H3N2)[a]	FJ798773.1
PB1[b]	99%	A/swine/Gent/96/2007(H3N2)	CY116448.1
		A/swine/Spain/82108/2007(H3N2)	CY116556.1
		A/swine/Spain/53207/2004(H1N1)	CY010586.1
PB2	99%	A/swine/Spain/82108/2007(H3N2)	CY116555.1
		A/swine/Gent/96/2007(H3N2)	CY116447.1
		A/swine/Hungary/13509/2007(H3N2)[a]	FJ798770.1
		A/swine/Spain/53207/2004(H1N1)	CY010587.1
PA	99%	A/swine/Spain/82108/2007(H3N2)	CY116557.1
		A/swine/Gent/96/2007(H3N2)	CY116449.1
	98%	A/swine/Hungary/13509/2007(H3N2)[a]	FJ798775.1
		A/swine/Spain/53207/2004(H1N1)	CY010585.1
NP	99%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161157.1
		A/swine/Spain/51915/2003(H1N1)	CY010575.1
		A/swine/Spain/53207/2004(H1N1)	CY010583.1
M	99%	A/swine/Spain/82108/2007(H3N2)	CY116561.1
		A/swine/Gent/96/2007(H3N2)	CY116453.1
	98%	A/swine/Ille et Vilaine/1455/1999(H1N1)	CY116383.1
NS	99%	A/swine/Spain/82108/2007(H3N2)[a]	CY116562.1
		A/swine/Gent/96/2007(H3N2)[a]	CY116454.1
		A/swine/Hungary/13509/2007(H3N2)[a]	FJ798774.1

	98%	A/swine/Spain/53207/2004(H1N1)	CY010584.1
80598LP1			
HA	98%	A/swine/Spain/54008/2004(H3N2)	CY010564.1
	97%	A/swine/Gent/96/2007(H3N2)	CY116450.1
NA	98%	A/swine/Spain/54008/2004(H3N2)	CY010566.1
	97%	A/swine/Damme/IDT5673/2006(H3N2)	GQ161148.1
PB1	98%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161154.1
		A/swine/Spain/53207/2004(H1N1)	CY010586.1
PB2	99%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161153.1
	98%	A/swine/Spain/53207/2004(H1N1)	CY010587.1
PA	99%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161155.1
		A/swine/Spain/53207/2004(H1N1)	CY010585.1
NP	98%	A/swine/Spain/42386/2002(H3N2)	CY020504.1
		A/swine/Ille et Vilaine/1455/1999(H1N1)	CY116381.1
		A/swine/Brno/00/2000(H1N1)	CY115882.1
M	99%	A/swine/Ploufragan/0214/2006(H1N2)	CY116542.1
		A/swine/Gent/132/2005(H1N1)	CY116437.1
		A/swine/Spain/53207/2004(H1N1)	CY010581.1
NS	98%	A/swine/Spain/54008/2004(H3N2)	CY010568.1
		A/swine/Spain/42386/2002(H3N2)[a]	CY020505.1
		A/swine/Cloppenburg/IDT4777/2005(H1N2)	EU053145.1
SF12091			
HA	99%	A/swine/Groitzsch/IDT6016-1/2007(H1N2)[a]	GQ161141.1
		A/swine/Kitzen/IDT6142/2007(H1N2)	GQ161145.1
		A/swine/Groitzsch/IDT6016-2/2007(H1N2)[a]	GQ161143.1
	98%	A/swine/Spain/40564/2002(H1N2)	CY116550.1
NA	99%	A/swine/Kitzen/IDT6142/2007(H1N2)	GQ161146.1
		A/swine/Groitzsch/IDT6016-1/2007(H1N2)	GQ161144.1
		A/swine/Groitzsch/IDT6016-2/2007(H1N2)	GQ161142.1
	98%	A/swine/Spain/40564/2002(H1N2)	CY116552.1
PB1	98%	A/swine/Spain/53207/2004(H1N1)	CY010586.1
		A/swine/Cloppenburg/IDT4777/2005(H1N2)	EU053139.1
		A/swine/Italy/71251/2005(H1N2)	JX843273.1
PB2	99%	A/swine/Gent/132/2005(H1N1)	CY116431.1
	98%	A/swine/Spain/53207/2004(H1N1)	CY010587.1
		A/swine/Italy/50568/2005(H1N2)	HQ845023.1
PA	99%	A/swine/Spain/53207/2004(H1N1)	CY010585.1
		A/swine/Italy/50568/2005(H1N2)	HQ709221.1
		A/swine/Italy/626-2/2006(H1N2)	HQ709222.1
NP	99%	A/swine/Gent/132/2005(H1N1)	CY116435.1
		A/swine/Spain/53207/2004(H1N1)	CY010583.1
M	99%	A/swine/Ploufragan/0214/2006(H1N2)	CY116542.1
		A/swine/Spain/51915/2003(H1N1)	CY010573.1
		A/swine/Gent/112/2007(H1N1)	CY116429.1
NS	99%	A/swine/Italy/159870-2/2005(H1N1)[a]	CY116483.1
	98%	A/swine/Spain/53207/2004(H1N1)	CY010584.1
80598LP4			
HA	94%	A/swine/England/690421/95(H1N2)	AF085415.1
		A/swine/Cotes d'Armor/790/1997(H1N2)	CY116410.1
		A/swine/Bakum/1832/2000(H1N2)	EU053148.1
NA[b]	96%	A/swine/England/88761/1997(H1N2)	CY116325.1
		A/swine/England/61605/1998(H1N2)	CY116246.1
	95%	A/swine/England/645913/1996(H1N2)	CY116262.1
PB1	98%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161154.1

		A/swine/Spain/53207/2004(H1N1)	CY010586.1
PB2	99%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161153.1
		A/swine/Spain/51915/2003(H1N1)	CY010579.1
PA	99%	A/swine/Greven/IDT2889/2004(H1N1)	GQ161155.1
	98%	A/swine/Spain/53207/2004(H1N1)	CY010585.1
NP	98%	A/swine/Spain/40564/2002(H1N2)	CY116551.1
		A/swine/Granstedt/IDT3475/2004(H1N2)	GQ161164.1
		A/swine/Haseluenne/IDT2617/2003(H1N1)	GQ161120.1
		A/swine/Spain/53207/2004(H1N1)	CY010583.1
M	99%	A/swine/Spain/51915/2003(H1N1)	CY010573.1
NS	99%	A/swine/Spain/51915/2003(H1N1)	CY010576.1

[a]Query coverage: 99%

[b] The sequence of the virus isolated in this study showed degenerate nucleotide. That means multiple nucleotide have been found at particular positions.

Table 3

Table 3: number of mutations in the antigenic sites of “Human-Like” H1 HA proteins.

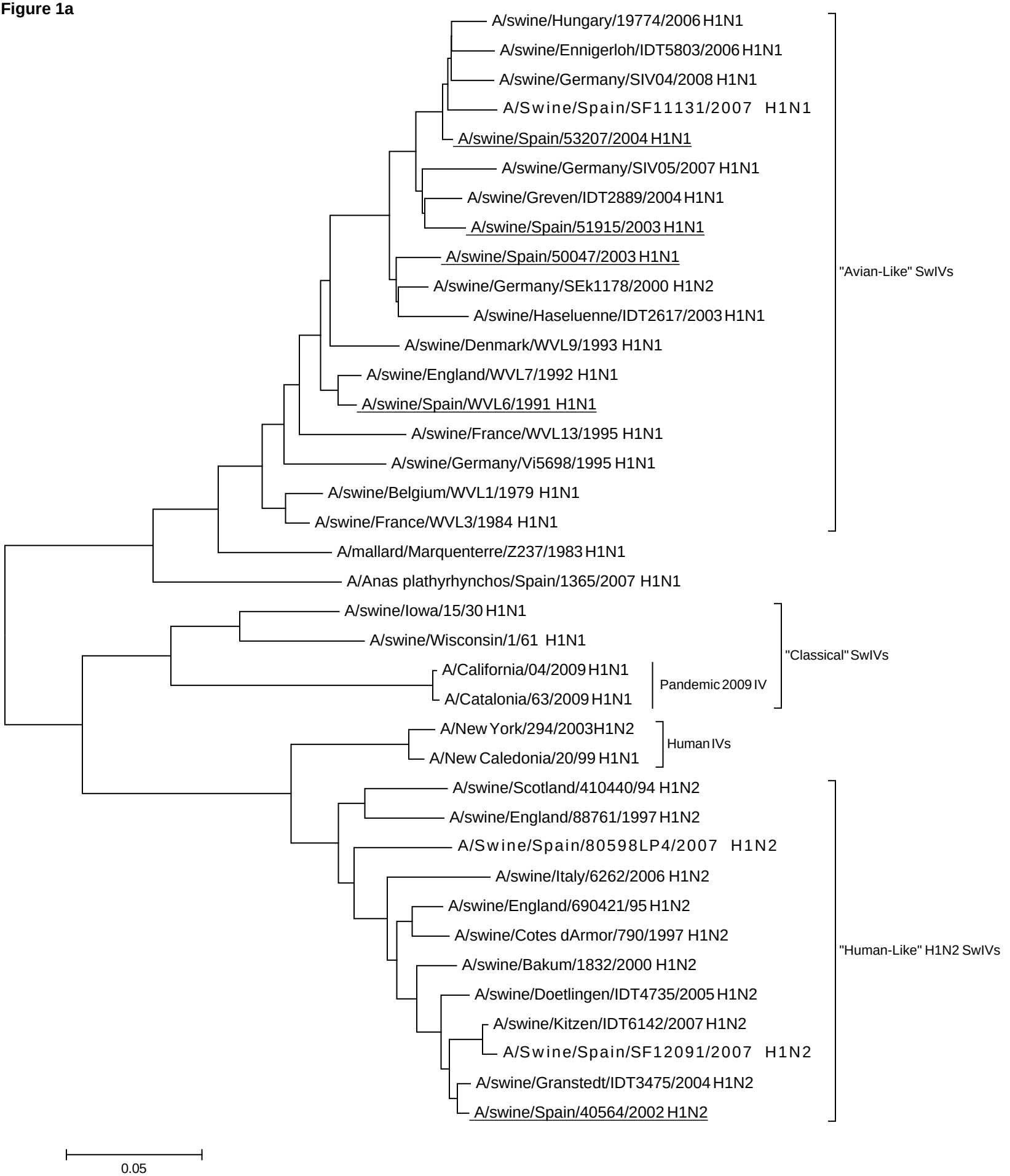
Strains	Antigenic sites			
	Ca	Sa	Cb	Sb
80598LP4-				
SF12091	6	1	0	1
A/Sw/Bakum/1832/00	5	0	0	1
SF12091-				
A/Sw/Bakum/1832/00	2	1	0	0

Table 4

Table 4. Glycosylation sites on the HA protein. The boldface positions are the glycosylation sites different in all strains.

Strain	Glycosylation sites positions
A/Swine/Spain/80598LP4/2007	27, 28, 40, **172, 177, 286**, 304, 498, 557
A/Swine/Spain/SF12091/2007	27, 28, 40, **172, 177, 286**, 304, 498, 557
A/swine/Bakum/1832/2000	27, 28, 40, **172, 177, 286**, 304, 498, 557

Figure 1a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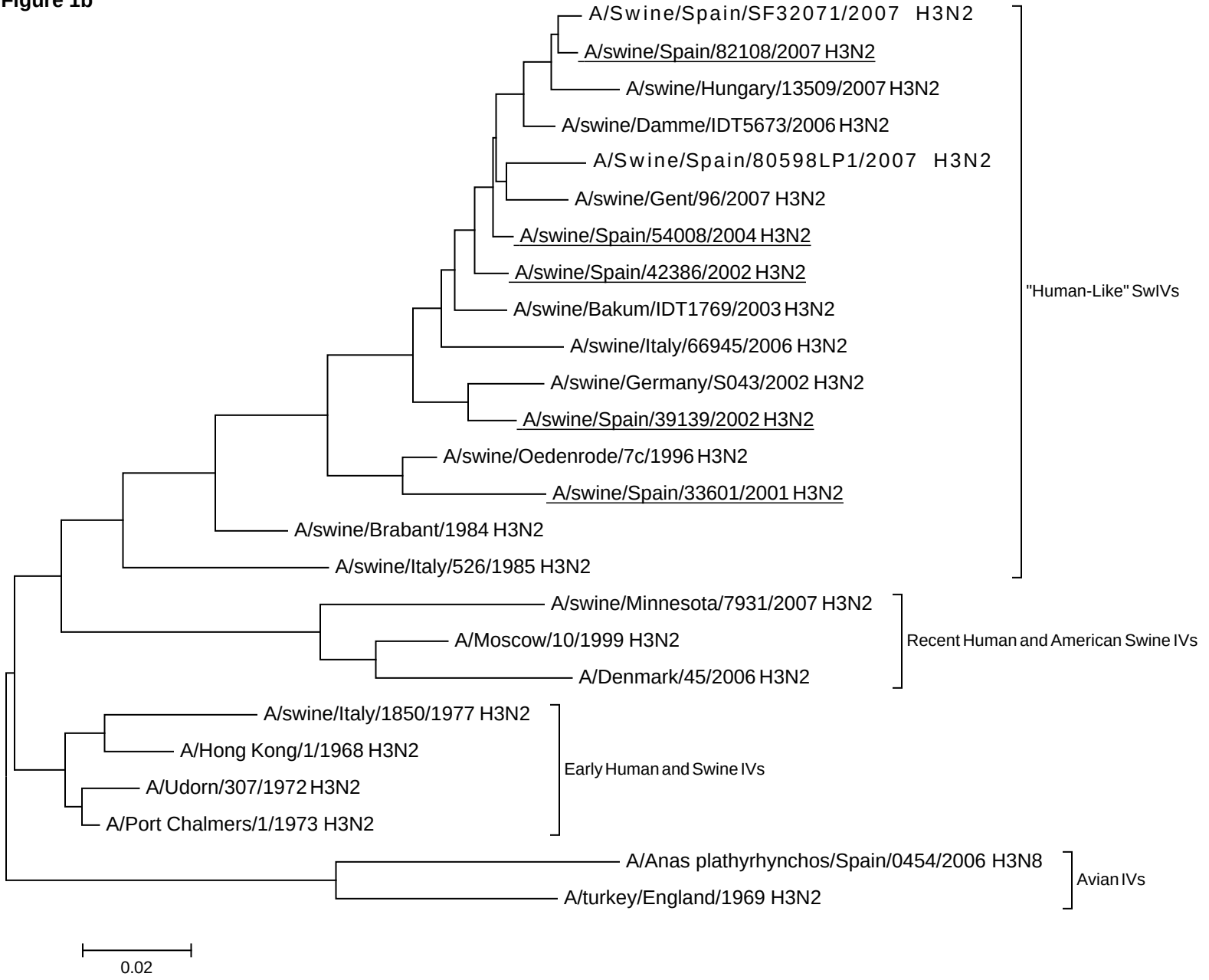
A/swine/Ennigerloh/IDT5803/2006 H1N1
A/swine/Germany/SIV04/2008 H1N1
A/Swine/Spain/SF11131/2007 H1N1
A/swine/Spain/53207/2004 H1N1
A/swine/Germany/SIV05/2007 H1N1
A/swine/Greven/IDT2889/2004 H1N1
A/swine/Spain/51915/2003 H1N1
A/swine/Spain/50047/2003 H1N1
A/swine/Germany/SEk1178/2000 H1N2
A/swine/Haseluenne/IDT2617/2003 H1N1
A/swine/Denmark/WVL9/1993 H1N1
A/swine/England/WVL7/1992 H1N1
A/swine/Spain/WVL6/1991 H1N1
A/swine/France/WVL13/1995 H1N1
A/swine/Germany/Vi5698/1995 H1N1
A/swine/Belgium/WVL1/1979 H1N1
A/swine/France/WVL3/1984 H1N1
A/mallard/Marquenterre/Z237/1983 H1N1
A/Anas plathyrhynchos/Spain/1365/2007 H1N1
A/swine/Iowa/15/30 H1N1
A/swine/Wisconsin/1/61 H1N1
A/California/04/2009 H1N1
A/Catalonia/63/2009 H1N1
A/New York/294/2003H1N2
A/New Caledonia/20/99 H1N1
A/swine/Scotland/410440/94 H1N2
A/swine/England/88761/1997 H1N2
A/Swine/Spain/80598LP4/2007 H1N2
A/swine/Italy/6262/2006 H1N2
A/swine/England/690421/95 H1N2
A/swine/Cotes dArmor/790/1997 H1N2
A/swine/Bakum/1832/2000 H1N2
A/swine/Doetlingen/IDT4735/2005 H1N2
A/swine/Kitzen/IDT6142/2007 H1N2
A/Swine/Spain/SF12091/2007 H1N2
A/swine/Granstedt/IDT3475/2004 H1N2
A/swine/Spain/40564/2002 H1N2
"Avian-Like" SwIVs
"Classical" SwIVs
Pandemic 2009 IV
Human IVs
"Human-Like" H1N2 SwIVs
0.05

Figure 1b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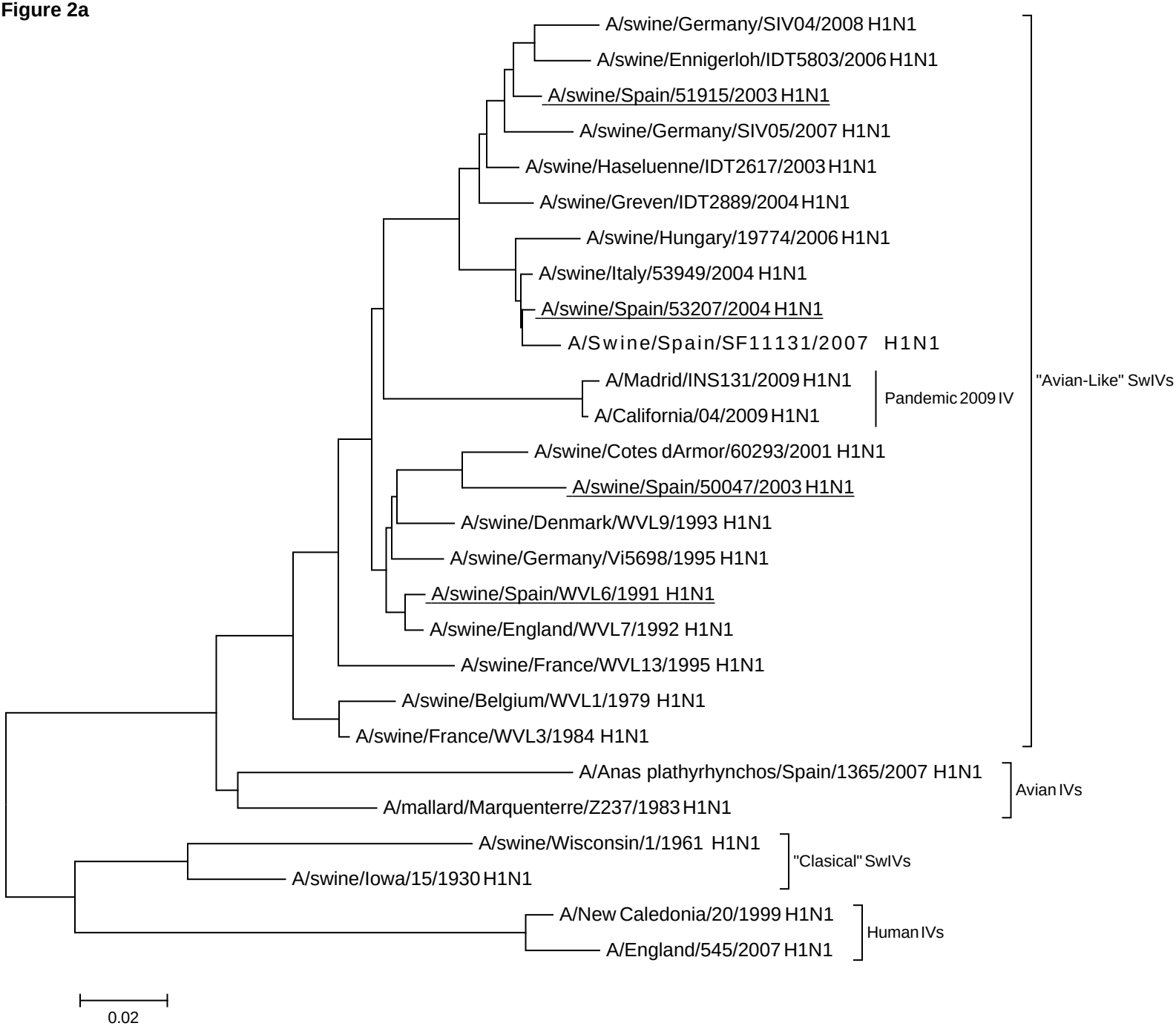
A/swine/Hungary/13509/2007 H3N2
A/swine/Damme/IDT5673/2006 H3N2
A/Swine/Spain/80598LP1/2007 H3N2
A/swine/Gent/96/2007 H3N2
A/swine/Spain/54008/2004 H3N2
A/swine/Spain/42386/2002 H3N2
A/swine/Bakum/IDT1769/2003 H3N2
A/swine/Italy/66945/2006 H3N2
A/swine/Germany/S043/2002 H3N2
A/swine/Spain/39139/2002 H3N2
A/swine/Oedenrode/7c/1996 H3N2
A/swine/Spain/33601/2001 H3N2
A/swine/Brabant/1984 H3N2
A/swine/Italy/526/1985 H3N2
"Human-Like" SwIVs
A/swine/Minnesota/7931/2007 H3N2
A/Moscow/10/1999 H3N2
A/Denmark/45/2006 H3N2
Recent Human and American Swine IVs
A/swine/Italy/1850/1977 H3N2
A/Hong Kong/1/1968 H3N2
A/Udorn/307/1972 H3N2
A/Port Chalmers/1/1973 H3N2
Early Human and Swine IVs
A/Anas plathyrhynchos/Spain/0454/2006 H3N8
A/turkey/England/1969 H3N2
Avian IVs
0.02

Figure 2a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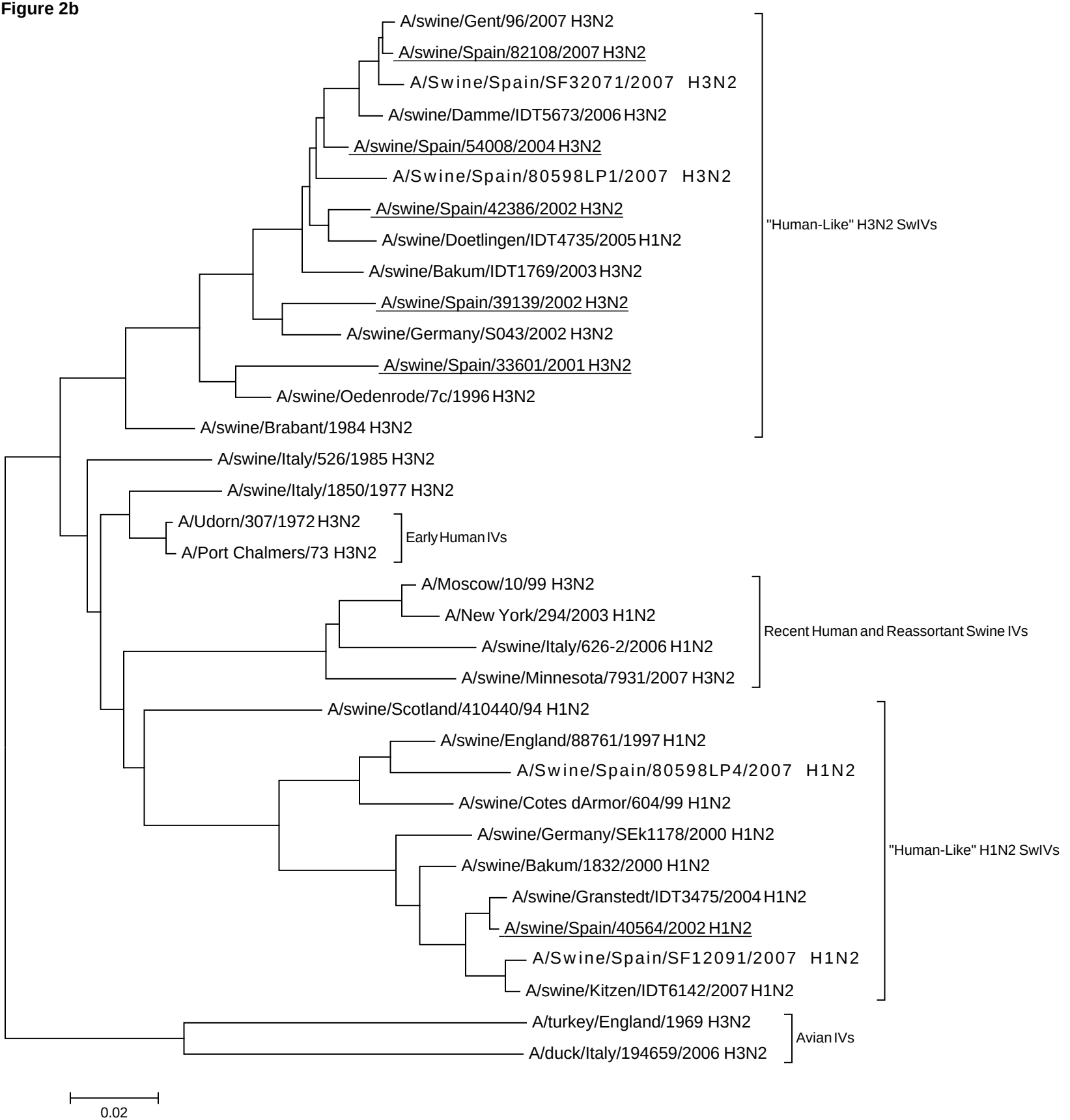
A/swine/Ennigerloh/IDT5803/2006 H1N1
A/swine/Spain/51915/2003 H1N1
A/swine/Germany/SIV05/2007 H1N1
A/swine/Haseluenne/IDT2617/2003 H1N1
A/swine/Greven/IDT2889/2004 H1N1
A/swine/Hungary/19774/2006 H1N1
A/swine/Italy/53949/2004 H1N1
A/swine/Spain/53207/2004 H1N1
A/Swine/Spain/SF11131/2007 H1N1
A/Madrid/INS131/2009 H1N1
A/California/04/2009 H1N1
Pandemic 2009 IV
"Avian-Like" SwIVs
A/swine/Cotes dArmor/60293/2001 H1N1
A/swine/Spain/50047/2003 H1N1
A/swine/Denmark/WVL9/1993 H1N1
A/swine/Germany/Vi5698/1995 H1N1
A/swine/Spain/WVL6/1991 H1N1
A/swine/England/WVL7/1992 H1N1
A/swine/France/WVL13/1995 H1N1
A/swine/Belgium/WVL1/1979 H1N1
A/swine/France/WVL3/1984 H1N1
A/Anas plathyrhynchos/Spain/1365/2007 H1N1
A/mallard/Marquenterre/Z237/1983 H1N1
Avian IVs
A/swine/Wisconsin/1/1961 H1N1
A/swine/Iowa/15/1930 H1N1
"Clasical" SwIVs
A/New Caledonia/20/1999 H1N1
A/England/545/2007 H1N1
Human IVs
0.02

Figure 2b


A/swine/Gent/96/2007 H3N2
A/swine/Spain/82108/2007 H3N2
A/Swine/Spain/SF32071/2007 H3N2
A/swine/Damme/IDT5673/2006 H3N2
A/swine/Spain/54008/2004 H3N2
A/Swine/Spain/80598LP1/2007 H3N2
A/swine/Spain/42386/2002 H3N2
A/swine/Doetlingen/IDT4735/2005 H1N2
A/swine/Bakum/IDT1769/2003 H3N2
A/swine/Spain/39139/2002 H3N2
A/swine/Germany/S043/2002 H3N2
A/swine/Spain/33601/2001 H3N2
A/swine/Oedenrode/7c/1996 H3N2
A/swine/Brabant/1984 H3N2
"Human-Like" H3N2 SwIVs
A/swine/Italy/526/1985 H3N2
A/swine/Italy/1850/1977 H3N2
A/Udorn/307/1972 H3N2
A/Port Chalmers/73 H3N2
Early Human IVs
A/Moscow/10/99 H3N2
A/New York/294/2003 H1N2
A/swine/Italy/626-2/2006 H1N2
A/swine/Minnesota/7931/2007 H3N2
Recent Human and Reassortant Swine IVs
A/swine/Scotland/410440/94 H1N2
A/swine/England/88761/1997 H1N2
A/Swine/Spain/80598LP4/2007 H1N2
A/swine/Cotes dArmor/604/99 H1N2
A/swine/Germany/SEk1178/2000 H1N2
A/swine/Bakum/1832/2000 H1N2
A/swine/Granstedt/IDT3475/2004 H1N2
A/swine/Spain/40564/2002 H1N2
A/Swine/Spain/SF12091/2007 H1N2
A/swine/Kitzen/IDT6142/2007 H1N2
"Human-Like" H1N2 SwIVs
A/turkey/England/1969 H3N2
A/duck/Italy/194659/2006 H3N2
Avian IVs
0.02

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